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Molecular diversity estimates of Pakistani citrus rootstocks

Ammara Noreen[1], Muhammad Asif[1], Waqar shafqat[2,*], Irfana Lalarukh[3], Bushra Sadia[4], Naseem Sharif[3] and Sufian Ikram[3]

[1]Horticultural Research station, Bahawalpur, Pakistan; [2]Department of Forestry, College of Forest Resources, Mississippi State University, Starkville 39759, Mississippi USA; [3] Department of botany, Government College Women University Faisalabad, Pakistan ;[3]Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan;[4]Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan
*Corresponding author's e-mail: ws805@msstate.edu

Quality rootstocks are mandatory to fortify and boost up the citrus industry. Traditionally, Pakistani citrus industry is based on two rootstocks (rough lemon and sour orange), and consequently facing many biotic and abiotic constraints. Characterization and evaluation of new rootstocks is also essential to expand the citrus genetic resources for better utilization and to improve future breeding programmes. In this study thirteen rootstocks were assessed based on twenty-five morphological traits (quantitative and qualitative) whereas genetic diversity was evaluated by using forty RAPD markers. Results manifested huge morphological diversity in Sachian Citromello, Citromello 1452, Yuma citrange and Benton rootstocks. Thirteen RAPD markers proved reliable and effective tool and showed significant amplification, producing a total of 286 fragments with 61.53% polymorphism. Moreover, RAPD markers showed the individuality of all the studied rootstocks and highlighted the similarities and dissimilarities among them. Findings of this study will provide basis for further investigations looking to the improvement of citrus rootstocks. Results are also valuable for future rootstock breeding programs, particularly in release of superior and trustworthy new rootstocks for Pakistani citrus industry. The combination of such kinds of morphological and molecular markers is highly powerful tool in accomplishing detailed analysis of Citrus phylogeny and origin.
Keywords: Characterization, screening, rootstocks, breeding, diversity, RAPD.

INTRODUCTION

Citrus rootstock and cultivars selection and improvement are most important to cover the loss due to biotic and abiotic stresses. There is a need to estimate the morphological and genetic diversity among the existing Citrus germplasm (Babar *et al.*, 2014). A complex taxonomic and phylogenic relationship among species and genera due to complicated reproductive biology, sexual compatibility among species as well as genera, polyembryony and high rate of bud mutation are observed in Citrus species (El-Mouei *et al*., 2011). Diversity and relationship among germplasm at morphology and genetic basis are important to evaluate the germplasm potential performance with respect to environment (Lowe *et al.*, 2004) and seems to be beneficial for rootstock improvement through breeding by Citrus breeders for the development of elite rootstocks and cultivars with desirable traits (Malik *et al.*, 2012).

Citrus industry of Pakistan is facing a lot of problems including biotic and abiotic stresses (Shafqat *et al*.,2019) which are severally limit overall plant growth and production. Rootstock is basic part of the citrus plant which controlled morphological, physiological, production and genetic characteristics of scion. So, it is a basic need for citriculture industry of Pakistan to work on some other rootstocks on the base of genetic and scion compatibility aspects to improve yield and plant adoptability to different environmental conditions. In this context, there was a dire need for morphological and genetic diversity estimation of citrus rootstocks. Genetic diversity estimation based on morphological characters has limitations as these are stage specific as well as largely affected by environmental factors (Malik *et al*., 2012). Molecular markers have used for varietal identification, distinguishing existing germplasm, gene mapping and gene cloning, polygenetic analysis, chromosome mapping and genetic diversity analysis (Gostimsky *et al*., 2005). RAPD is a PCR based marker system which has been used to assess genetic diversity among different citrus cultivars like pummelo, lemon, mandarin, oranges and grapefruit (Cai *et al*., 2007; Golein *et al*., 2012).

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RAPD markers are most simple primers to assess genetic diversity as it contains 10 mer random primers, which don't require any preceding information of DNA sequence data for their designing (Shahzadi *et al*., 2016). As DNA is distributed in whole genome, these are easy to be analyzed. In addition, lesser cost, infrastructure and DNA concentration requirement make their use so frequently (Sarwat *et al*., 2011). In *Citrus*, markers have also been established at DNA level to distinguish the collected germplasm (Biswas *et al*., 2010) and to study phylogenetic and taxonomic relationships among different *Citrus* genera and species (Nhan *et al*., 2002; Naz *et al*., 2014) and selection for its further use in breeding program (Sharma *et al*., 2015).

Considered the importance of citrus rootstocks current study was planned for the assessment of morphological and genetic diversity of Citrus rootstocks. This study will be helpful for laying the genetic basis of citrus rootstocks in Pakistan and facilitate breeding for improved characters.

MATERIALS AND METHODS

***Plant material*:** Ten citrus rootstocks (Sour orange, Kharnakhatta, Sachian Citromello, Chakotra, Brazillian sour orange, Citromello 1452, Benton, Bitter sweet orange, Yuma citrange and Rough lemon) were selected from experimental fruit orchard (square # 9) of Institute of horticultural sciences, University of Agriculture Faisalabad, Pakistan. Selected fruit trees were 10-12 years old, healthy, disease free and with vigorous growth conditions.

***Analysis of morphological traits*:** Morphological characterization regarding tree and leaf were studied following IPGRI citrus plant descriptors (1999) as standards. Twenty five vegetative morphological traits of leaf and tree were used to group each rootstock including Trunk surface (score), Tree shape (score), Tree growth habit (score), Density of branches (score), Branch angle (score), Spine density (score), Spine length (cm), Spine shape (score), Shoot tip color (score), Shoot tip surface (score), Leaf Vegetative life cycle (score), Leaf division (score), Intensity of green color of leaf blade (score), Leaf color variegation (score), Leaf lamina attachment (score), Leaf lamina length (cm), Leaf lamina width (cm), Ratio leaf lamina length/width (cm), Leaf lamina shape (score), Leaf lamina margin (score), Leaf apex (score), Absence/presence of petiole wings (score), Petiole wing width (cm), Petiole wing shape (score) and Junction between petiole and lamina (score).

***Statistical analysis*:** Multivariate analysis of variance (i.e., principal component analysis and cluster analysis) was performed by grouping the selected citrus rootstocks based on similarities for morphological attributes with XLSTAT (2018.1) software. Correlation coefficients were calculated for studied characters to choose useful traits for effective indirect selection and to minimize ineffective characters and the construction of relevant PCA plots were buildup.

***DNA extraction*:** Young fully matured healthy green leaves were collected from selected citrus rootstocks for DNA extraction. DNA extraction was done following CTAB method (Altaf *et al*., 2014) with some modifications. Leaves were washed with distilled water to remove dust particles or debris, dried and then grinded into fine powder in 2X CTAB. Then pre-heated CTAB along with 1% Mercaptoethanol was added to the tissues and incubated for 30-50 min at 65°C

Table 1. List of RAPD primers

Sr. No.	Primer Name	Sequence	Sr. No.	Primer Name	Sequence
1.	A-01	CAGGCCCTTC	2.	L-01	GGCATGACCT
3.	A-02	TGCCGAGCTG	4.	L-02	TGGGCGTCAA
5.	C-01	TTCGAGCCAG	6.	L-03	CCAGCAGCTT
7.	C-02	GTGAGGCGTC	8.	L-04	GACTGCACAC
9.	C-03	GGGGGTCTTT	10.	L-05	ACGCAGGCAC
11.	C-04	CCGCATCTAC	12.	L-06	GAGGGAAGAG
13.	C-06	GAACGGACTC	14.	L-07	AGGCGGGAAC
15.	C-07	GTCCCGACGA	16.	L-08	AGCAGGTGGA
17.	C-08	TGGACCGGTG	18.	L-09	TGCGAGAGTC
19.	C-09	CTCACCGTCC	20.	L-10	TGGGAGATGG
21.	C-10	TGTCTGGGTG	22.	L-11	ACGATGAGCC
23.	I-1	ACCTGGACAC	24.	L-12	GGGCGGTACT
25.	I-02	GGAGGAGAGG	26.	L-13	ACCGCCTGCT
27.	I-03	CAGAAGCCCA	28.	L-14	GTGACAGGCT
29.	I-04	CCGCCTAGTC	30.	L-15	AAGAGAGGGG
31.	I-05	TGTTCCACGG	32.	L-16	AGGTTGCAGG
33.	I-07	CAGCGACAAG	34.	L-17	AGCCTGAGCC
35.	I-08	TTTGCCCGGT	36.	L-18	ACCACCCACC
37.	I-09	TGGAGAGCAG	38.	L-19	GAGTGGTGAC
39.	I-10	ACAACGCGAG	40.	L-20	TGGTGGACCA

followed by addition of chloroform:isomylalcohal (24:1). This aqueous phase was subjected to centrifugation @ 13000 rpm for 13-15 min at room temperature. In order to precipitate DNA, 700-800 µl of 60% chilled ethanol was added and mixed. The mixture was centrifuged @13000 rpm for about 15 min to precipitate DNA and then supernatant was discarded and resultant pellet was washed, dried out and resuspended in d_3H_2O. To further purify the DNA, RNase was added@ 1µl of stock RNase/20 µl of DNA solution and incubated for 1hour at 37ºC. The DNA was treated again with chloroform/isoamyl alcohol and mixed gently followed by centrifugation for 10 min@1300 rpm. Then 3M NaCl was added in tubes. Chilled isopropanol was added @66% (by volume) to precipitate DNA. DNA was pelleted by centrifugation for 5 minutes at 1300 rpm then 70% ethanol was added and DNA pellet was dissolved in 100µl d_3H_2O. 0.8% agarose gel electrophoresis was used to check Genomic DNA quality. Furthermore, quantification was done by using Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). DNA samples were diluted to approximately 15 ng/µl DNA.

***Semi qPCR for RAPD analysis*:** PCR reaction was carried out with following combination; template DNA (2.5 µl), distilled water (10.8 µl), Primer (2 µl @30ng/µl), dNTPs (4 µl @10mM), $MgCl_2$ (3 µl @25mM), *Taq*DNA polymerase (0.2 µl), Taq polymerase buffer (2.5 µl @ 10X) in a thermal cycler (Eppendorf AG No. 533300839, Germany). PCR reaction conditions consisted of denaturation at 94 °C for 5 min, followed by annealing at 36°C for 1 min, then elongation at 72 °C for 1 min and final elongation a cycle of 1 min at 72 °C. PCR products were electrophoresed at 1.2 % agarose gel stained with 0.55 µg / ml ethidium bromide. Then gel was observed by UV Transilluminator (BioRAD, ChemiDoc, XPS+ USA) to examine banding pattern under UV light and then photographed with Dolphin del documentation.

***Scoring and data analysis*:** Clear and repeatable amplified products were scored as 0 (absent) and 1 (present) band to make a data matrix. The data matrix was analyzed to calculate the genetic distance and genetic similarity using Popgene software ver. 1.32 (Yeh*et al*.,2000). The phylogenetic tree was made using distance matrix with Nei's unweighed pair group of arithmetic means (UPGMA).

RESULTS

***Principle component analysis (Morphological traits*):** PCA among 13 citrus rootstocks based on 25 five morphological traits was studied which accounted 26.52%, 19.77% and 16.77% for first three factors respectively. These results predicted that leaf vegetative cycle (LVC), leaf division (LD) and branch angle (BA) had highly positive loadings whereas the leaf lamina length (LLL) and leaf lamina width (LLW) had highly negative loadings in the PC1 axes (Table 2).

Scree plot showed that each principal component had a role in variation as showed by lower line (Fig. 1). F1 had the highest proportion in cumulative variability (>80%), followed by F2 (>60%) and F3 (>50%). Rest of the factors observed with little contribution in cumulative variability.

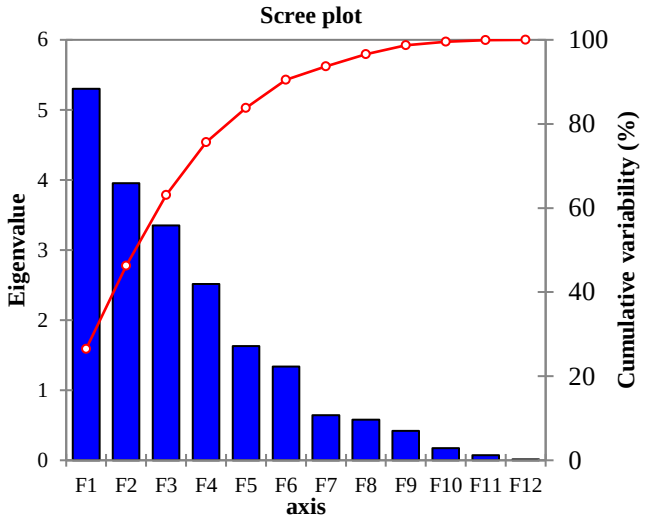

Figure 1. Scree plot shows the eigenvalues on the y-axis and the number of factors on the x-axis, Upper line in figure showed the cumulative variance (%) explained by the components.

Table 2. Eigenvalues, variation proportion and eigenvectors linkage with first three axes of the PCA in 13 citrus rootstocks.

Axe	1	2	3
Eigenvalue variation	5.303	3.955	3.353
Variance proportion			
Individual %	26.516	19.773	16.767
Cumulative %	26.516	46.289	63.057
Eigenvectors*	0.411 LVC	0.435 PW	0.466 DOF
	0.411 LD	0.413 PWW	0.356 IGC
	0.357 BA	0.362 PWS	0.220 LLS
	-0.376 LLL	-0.215 LLS	-0.372 LLM
	-0.331 LLW	-0.214 SL	-0.256 P/L

*Only parameters with high loadings in three principal components were shown.

Major groups can be easily identified from scatter plot, PCA and Biplot representation of rootstcocks and parameters for axes among 1-2 and 1-3 (Fig. 2). The PCA results showed that the first axis opposed trifoliate rootstocks (Sachian Citromello, Citromello 1452, Benton and Yuma citrange) from all other citrus rootstocks. These are characterized by leaf division (trifoliate), leaf vegetative life cycle, branch angle and spine density but others are characterized by monofoliate leaves and evergreen leaf vegetative life cycle. These are characterized by leaf division (trifoliate), leaf vegetative life cycle, branch angle and spine density but others are characterized by monofoliate leaves and evergreen leaf vegetative life cycle (Fig. 2). In axes 1-3 showed that trifoliate rootstocks (Sachian Citromello, Citromello 1452, Benton and Yuma citrange) opposes from all other citrus rootstocks especially Brazillian Sour Orange and Sour orange. Kharna Khatta and *Citrus obovoidea* opposes to Bitter sweet orange, Galgal and Rough lemon (Fig. 2). The second component analysis opposed Chakotra, Gadadahi, Brazillian sour orange, Sour Orange. The first cultivars group has

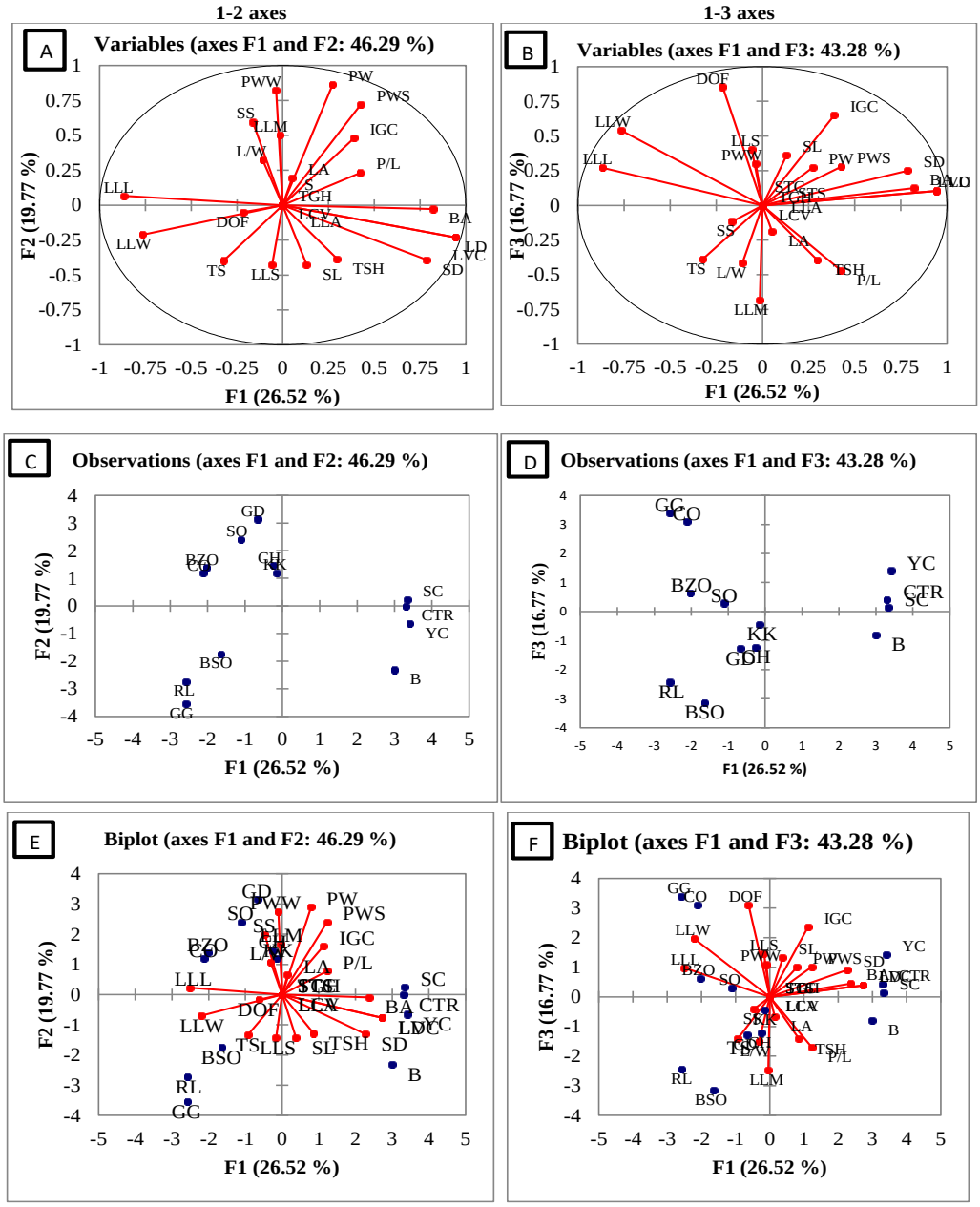

Figure 2. Scatter plot in relation to axes 1-2 (A) and axes 1-3 (B), Graphic representation the citrus genotypes axes 1-2 (C) and axes 1-3 (D), Biplot representing the citrus genotypes axes 1-2 (C) and axes 1-3 (D).

positive correlation with the second component analysis, so they are characterized by leaf division (monofoliate) and evergreen leaf vegetative life cycle. In axes 1-3Brazillian sour orange, Sour Orange, Galgaland *Citrus obovoidea*to Bitter sweet orange, KharnaKhatta, Chakotra, Gadadahi and Rough lemon. The first cultivars group has positive correlation with the second component analysis, so they are characterized by leaf division (monofoliate) and evergreen leaf vegetative life cycle (Fig. 2).

***Correlations (Morpholigical traits*):** The correlation matrix between explored characters showed that leaf lamina length (LLL) exibited significant positive correlation with leaf lamina width (LLW) while it showed negative correaltion with tree shape (TSH), branch angle (BA), leaf division (LD) and leaf vegetative life cycle (LVC). Branch angle (BA) exibited significant positive correlation with spine density (SD), leaf division (LD) and leaf vegetative life cycle (LVC). Leaf lamina width (LLW) showed significant correlation with density of branches (DOF) while it exibited negative correlation with leaf division (LD) and leaf vegetative life cycle (LVC) (Table 3). Leaf division (LD) had highly sigificant correlation with leaf vegetative life cycle (LVC) and spine density (SD) while spine density (SD) had significant correlation with spine length (SL) and leaf vegetativ life cycle (LVC). Leaf lamina width (LLW) also exibited negative correlation with leaf lamina length (LLA) and junction between leaf lamina and petiole (P/L). Leaf apex (LA) with ratio between leaf lamina length (LLA) and width (LLW) (Table 3).

***Dendrogram construction (By means of Aglomerative hirarichal clustering)*:** Dendogram construction based on morphological data succefully divided the 13 citrus rootstocks into two distinct clusters i.e., C-1 and C-2 (Fig. 3). Chakotra was placed in C-1 and all other genotypes were grouped under C-2 that was further subdivied into two subclusters i.e., C-2A and C-2B. Benton, Yuma Citrange, Sachian Citromello and Citromello 1452 were categorized in C-2A and observed all genotypes with trifoloiate genotypes, perhaps that was a major feature to group them jointly. All other rootstocks were grouped under C-2B, which was further divided into two subclusters i.e., C-2B_1 and C-2B_2.

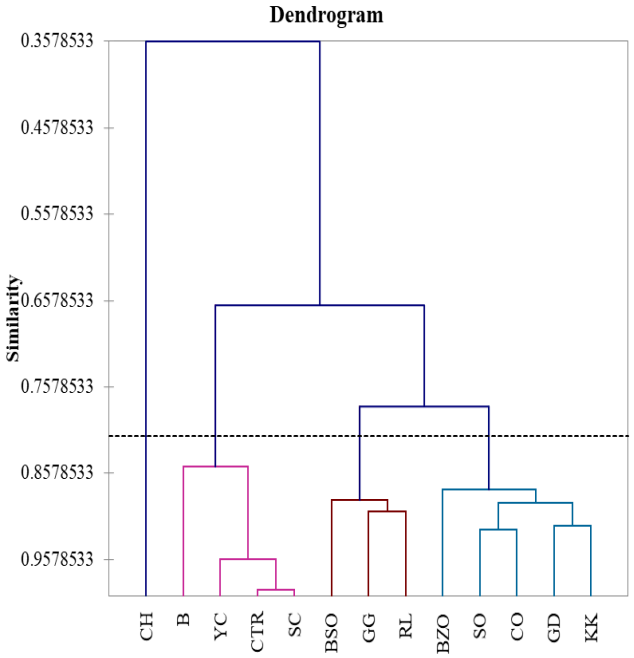

Figure 3. Dendrogram of 13 citrus rootstocks based on morphological data, CH (Chakotra), B (Benton, YC(Yuma citrange), CTR (), SC (Sachitan citrumello), BSO (Bitter sweet orange), GG (Galgal), RL (Rough lemon), BZO (Brazilian sour orange), SO (Sour orange), CO (Citrumello 1452), GD (Gadha dahi), KK (Kharna khatta).

Table 3. Correlation Matrix

	TS	TSH	DOF	BA	SD	SL	SS	LVC	LD	IGC	LLL	LLW	L/W	LLS	LLM	LA	PW	PWW	PWS
TSH	0.123																		
DOF	-0.047	-0.380																	
BA	-0.228	-0.101	-0.128																
SD	0.000	0.272	0.207	0.605*															
SL	0.067	0.223	0.528	-0.055	0.593*														
SS	-0.123	-0.409	-0.069	0.101	-0.544	-0.545													
LVC	-0.192	0.284	-0.108	0.843*	0.850*	0.155	-0.284												
LD	-0.192	0.284	-0.108	0.843*	0.850*	0.155	-0.284	1.000**											
IGC	-0.677*	-0.182	0.519	0.337	0.272	0.223	0.182	0.284	0.284										
LLL	0.290	-0.562*	0.504	-0.664*	-0.510	0.118	0.035	-0.794*	-0.794*	-0.192									
LLW	0.254	-0.358	0.594*	-0.508	-0.382	0.090	-0.071	-0.592*	-0.592*	-0.127	0.788*								
L/W	-0.028	-0.096	-0.135	-0.247	-0.147	0.218	0.103	-0.273	-0.273	-0.010	0.219	-0.391							
LLS	-0.366	-0.296	0.304	0.115	0.077	0.112	-0.039	0.070	0.070	0.039	0.093	0.233	-0.210						
LLM	0.075	0.129	-0.504	-0.150	-0.330	-0.452	0.350	-0.202	-0.202	-0.111	-0.152	-0.475	0.444	-0.418					
LA	-0.101	0.150	-0.057	-0.157	0.075	0.524	-0.150	-0.107	-0.107	0.150	0.084	-0.322	0.796*	-0.365	0.058				
PW	-0.433	-0.284	0.108	0.184	0.000	-0.155	0.284	0.083	0.083	0.640*	-0.067	-0.186	0.088	-0.463	0.202	0.202			
PWW	-0.366	-0.262	0.176	-0.109	-0.277	-0.362	0.262	-0.139	-0.139	0.540	0.132	0.066	0.004	-0.441	0.206	0.030	0.844*		
PWS	-0.390	-0.238	0.152	0.260	0.225	-0.041	0.238	0.245	0.245	0.576*	-0.179	-0.274	0.014	-0.284	0.152	0.073	0.900**	0.637*	
P/L	0.123	-0.182	-0.380	0.337	0.272	-0.099	0.182	0.284	0.284	-0.182	-0.279	-0.638*	0.389	-0.129	0.368	0.150	0.178	-0.162	0.413

*=Values are significant at α=0.05 , **=Values are highly significant at α=0.01

Bitter sweet orange, Galgal and Rough lemon showed more resemblance based on morphological characters and categorized together in C-$2B_1$ whereas five rootstocks including Brazilian sour orange, Sour orange, Citrumello 1452, Gadha dahi and Kharna khatta were stayed jintly in C-$2B_2$. It was also observed that Grape fruit and Kharna khatta were at highest dissimilarity based on the observed morphological traits (Fig. 3).

***Genetic studies based on RAPD markers*:** Forty RAPD primers were used to analyze DNA of 10 citrus rootstocks, out of which thirteen gave fruitful results, producing 286 fragments, with varying intensity and size. Almost 110 bands were monomorphic and rests were polymorphic (Fig. 4). Number of bands varied with changing genotype and primer. RAPD markers identified a polymorphism of about 61.53% among citrus rootstocks. Recorded variation in number of amplified fragments among studied primes was ranged from 9 to 54. Highest number of fragments was amplified by Primer GL Decamer L12 while lowest number of bands was produced by GL Decamer L16 (Fig. 4). Variation in number of amplified fragments among different rootstocks with an average of 29.2 fragments was observed. Highest number of amplified fragments was found for Yuma citrange and Citromello 1452 while lowest number of fragments were recorded for Brazilian sour orange (Fig. 1). Amplification on the gel electrophoresis of four RAPD profile generated by Primer GLL15, GLL16, GLL08 and L12 are presented in Figure 2 (A-D) for ten rootstocks. Highest amplified bands observed for Yuma citrange while lowest for Brazilian sour orange (Fig. 5).

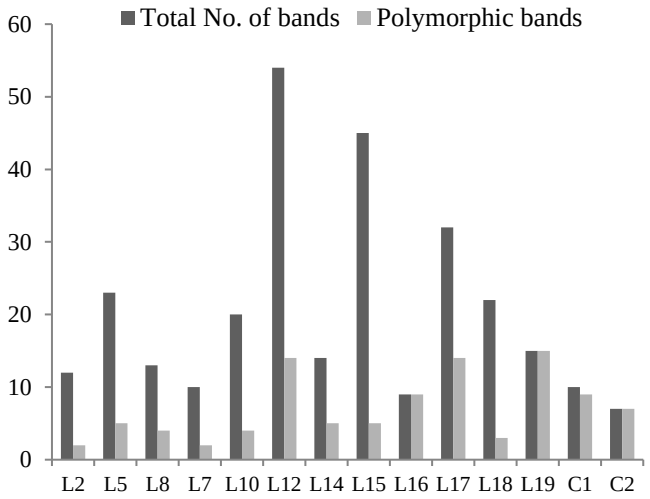

Figure 4. Comparison of total number of bands and polymorphic bands per primer.

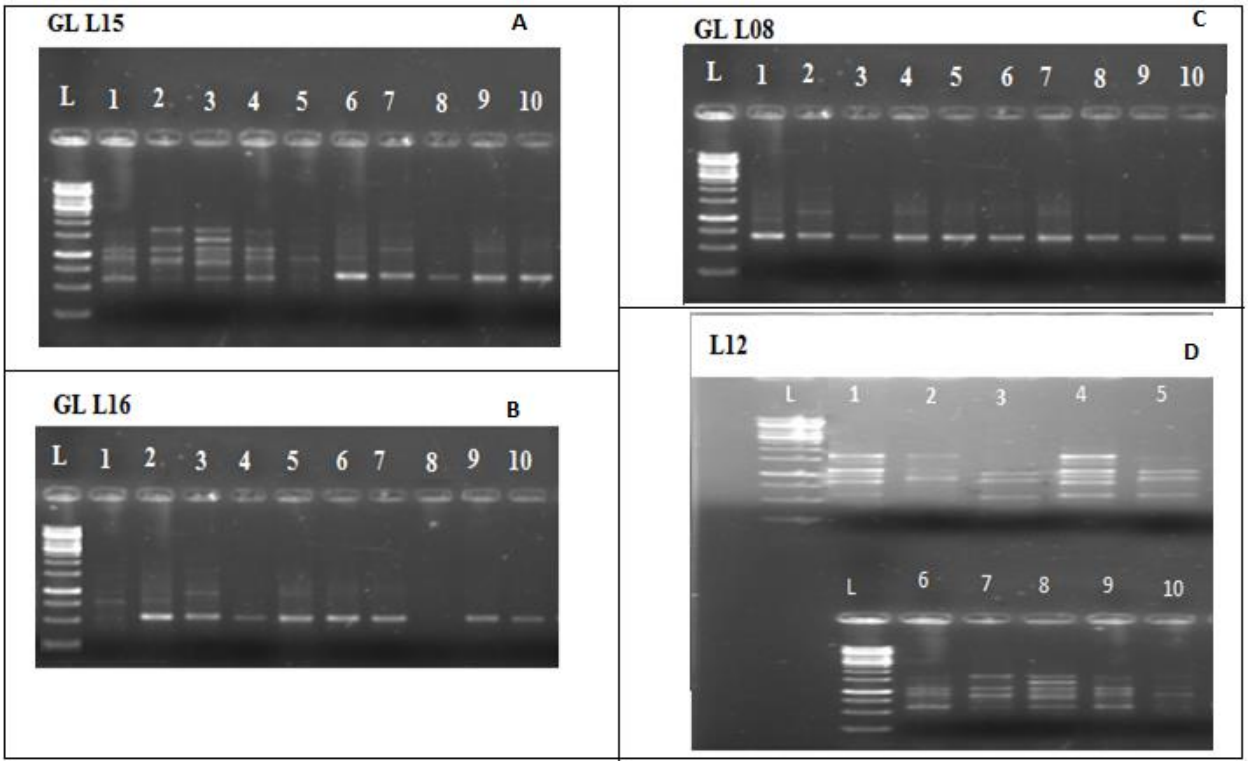

Figure 5. RAPD profile generated by Primer GL L15 (Fig 2A), GL L16 (Fig 2B) GL L08 (Fig 2C) and L12 (2D). L: Ladder, 1: Sour orange, 2: Kharnakhatta, 3: Sachian Citromello, 4: Chakotra, 5: Brazilian sour orange, 6: Citromello-1452, 7: Benton, 8: Bitter sweet orange 9: Yuma citrange and 10: Rough lemon.

Table 4. Similarity matrix of 10 citrus rootstocks.

	Sour Orange	Karna Khatta	Chakotra	Brazillian Sour Orange	Bitter Sweet Orange	Rough Lemon	Benton	C.1452	Y. Citrange	S. Citromello
Sour Orange	1.000	0.648	0.704	0.685	0.722	0.685	0.537	0.611	0.611	0.500
Karna Khatta		1.000	0.685	0.630	0.556	0.593	0.482	0.519	0.482	0.593
Chakotra			1.000	0.722	0.722	0.611	0.611	0.611	0.574	0.574
Brazillian Sour				1.000	0.778	0.667	0.593	0.556	0.556	0.556
Bitter Sweet					1.000	0.667	0.556	0.556	0.556	0.444
Rough Lemon						1.000	0.593	0.630	0.519	0.556
Benton							1.000	0.741	0.741	0.741
C.1452								1.000	0.815	0.741
Y. Citrange									1.000	0.704
S. Citromello										1.000

Nei's unbiased measures of Genetic similarity [Nei (1978) Genetics 89:583-590]

Table 5. Similarity matrix of 10 citrus rootstocks.

	Sour Orange	Karna Khatta	Chakotra	Brazilian sour O	Bitter Sweet O	Rough Lemon	Benton	C.1452	Y. Citrange	S. Citromello
Sour Orange	1.000									
Karna Khatta	0.434	1.000								
Chakotra	0.351	0.378	1.000							
Brazillian sour	0.378	0.463	0.325	1.000						
Bitter Sweet	0.325	0.588	0.325	0.251	1.000					
Rough Lemon	0.378	0.523	0.493	0.406	0.406	1.000				
Benton	0.622	0.731	0.493	0.523	0.588	0.523	1.000			
C.1452	0.493	0.657	0.493	0.588	0.588	0.463	0.300	1.000		
Y. Citrange	0.493	0.731	0.555	0.588	0.588	0.657	0.300	0.205	1.000	
S. Citromello	0.693	0.523	0.555	0.588	0.811	0.588	0.300	0.300	0.351	1.000

Nei's unbiased measures of Genetic Distance: genetic identity

***Genetic similarities and relationships among citrus rootstocks*:** Multivariate analysis was performed based on UPGMA to assess genetic similarity among citrus rootstock germplasm using Popgene software (Table 4). Nei's similarity indices showed that these rootstocks possess a genetic similarity of 44.44-81.48%. Citromello 1452 and Yuma citrange showed the maximum genetic similarity (81.48%) while Bitter Sweet Orange and Sachian Citromello exhibited the minimum genetic similarity (44.44%) (Table 5).

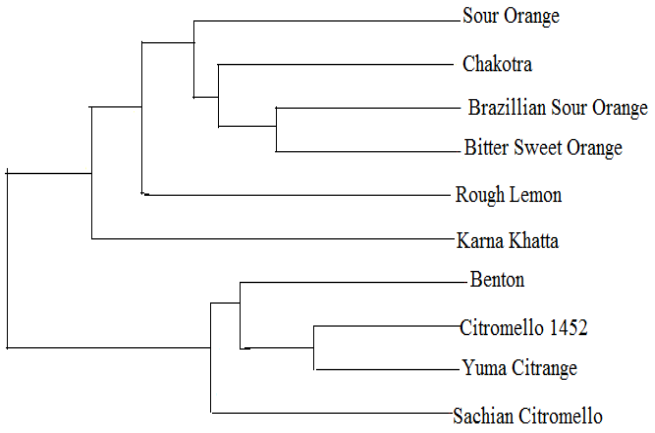

Figure 6. Dendrogram of 10 citrus rootstocks based on RAPD analysis.

***Cluster analysis based on RAPD analysis*:** Dendrogram was generated based on RAPD studies and two major clusters were produced i.e., C1 and C2. Cluster C1 contained four rootstocks i.e., Benton, Yuma Citrange, Citromello 1452 and Sachian Citromello while C2 was consisted of remaining six rootstocks (Fig. 6) and further distributed into two clusters C2A and C2B. C2A consists of only one rootstock i.e., Kharna Khatta and C2B consisted on five rootstocks including Sour Orange, Chakotra, Brazilian Sour Orange and Bitter Sour Orange. Sachian Citrumello and Sour Orange were found to be most diverse based on genetic characterization studies.

DISCUSSION

Characterization plays significant role in germplasm identification in order to facilitate the conservation, utilization and breeding of plant germplasm. Morphological markers have been used to differentiate accessions since decades. Although they are affected by the many environmental factors, but they have vital importance in diversity estimation (Elameen*et al*., 2010). Morphological analysis had been used to estimate diversity between Pakistani citrus species like Kinnow mandarin and rough lemon by Jaskani *et al.*, 2006 and Altaf and Khan, 2008. Variations in Himalayan citrus were also studied based on morphological markers (Sharma *et al*., 2004).

In order to aid convenience in studying agronomic traits morphological markers are widely used, as their study is cheaper and easier to conduct. Although morphological and molecular diversity estimation is independent of each other but in case of citrus these complement to genetic variation studies but in mandarin morphological characterization is independent of genetic variation (Koehler-santos *et al*., 2003; Campos *et al*., 2005). Polygenetic characters are successfully explained and identified by morphological markers. This study revealed the broad range of variability in measured morphological parameters. Sachian Citromello, Citromello 1452, Yuma citrange and Benton rootstocks showed trifoliate leaves and deciduous leaf vegetative life cycle as compared to other rootstocks, which have monofoliate leaves and evergreen leaf vegetative life cycle. Principal component analysis showed that these traits like intensity of green color of leaves, leaf division, leaf vegetative life cycle, branch angle, leaf lamina length and width, ratio of leaf lamina length and width, absence or presence of petiole wing, petiole wing shape and petiole wing width possessed a greater proportion of the observed variability. Whereas, Pearson's coefficient correlation showed that a significant positive and negative correlation between all the recorded parameters of citrus rootstocks. Morphological diversity in thirteen citrus rootstocks was estimated by the use of PCA analysis. The results depicted great variation in twenty-five selected morphological traits and were similar to the results of Pearson's coefficient correlation.

Forty RAPD markers were also used to assess genetic variation among 10 citrus rootstocks. Out of forty markers, 13 markers showed amplification, producing a total of 286 fragments. Genetic distance and genetic similarity were assessed and neighbor joining phylogenetic tree was constructed using distance matrix with unweighed pair group method with arithmetic average.

Polymorphic band are similar to the reports of various scientists like Filho *et al*. (2000) found about 71.43% polymorphism with a total of 112 amplification products with an average of 6.63 bands per primer. He reported that highest number of bands were generated by GLC-19, GLA-9 and GLK-7. Nhan *et al*. (2002) reported the polymorphism percentage of 65.83%. Polymorphism using 16 RAPD primers among citrus varieties. El-Moue*et al*. (2011) also found a polymorphism of 80.63% with a total of 143 bands in 31 citrus genotypes. This level of polymorphism shows a higher degree of divergence in citrus genotypes.

***Conclusion*:** Overall it was concluded that Sachian Citromello, Citromello 1452, Yuma citrange and Benton rootstocks showed trifoliate leaves and deciduous leaf vegetative life cycle. Sachian Citrumello and Sour Orange were found to be most diverse based on genetic characterization studies. Citromello 1452 and Yuma citrange showed the maximum genetic similarity (81.48%) while Bitter Sweet Orange and Sachian Citromello exhibited the minimum genetic similarity (44.44%). It was also observed that Grape fruit and Kharna khatta were at highest dissimilarity based on the observed morphological traits.

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